

2008

# Androgen and Estrogen ( $\alpha$ ) Receptor Localization on Periaqueductal Gray Neurons Projecting to the Rostral Ventromedial Medulla in the Male and Female Rat

Dayna R. Loyd

Anne Z. Murphy PhD

Georgia State University, amurphy@gsu.edu

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## Recommended Citation

Loyd, D. R., & Murphy, A. Z. (2008). Androgen and estrogen ( $\alpha$ ) receptor localization on periaqueductal gray neurons projecting to the rostral ventromedial medulla in the male and female rat. *Journal of Chemical Neuroanatomy*, 36(3-4), 216-226.  
doi:<http://dx.doi.org/10.1016/j.jchemneu.2008.08.001>

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Manuscript Number: CHENEU-D-08-00030R1

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Article Type: Research Article

Section/Category:

Keywords: gonadal steroid receptor; hormone; immunohistochemistry; sex differences; descending modulatory pathway; pain; antinociception

Corresponding Author: Dr. Anne Z Murphy, Ph.D.

Corresponding Author's Institution: Georgia State Univ

First Author: Dayna R Loyd, MS

Order of Authors: Dayna R Loyd, MS; Anne Z Murphy, Ph.D.

Manuscript Region of Origin:

**Abstract:** The periaqueductal gray (PAG) is involved in many gonadal steroid-sensitive behaviors, including responsiveness to pain. The PAG projects to the rostral ventromedial medulla (RVM), comprising the primary circuit driving pain inhibition. Morphine administered systemically or directly into the PAG produces greater analgesia in male compared to female rats, while manipulation of gonadal hormones alters morphine potency in both sexes. It is unknown if these alterations are due to steroidal actions on PAG neurons projecting to the RVM. The expression of androgen (AR) and estrogen (ER $\alpha$ ) receptors in the PAG of female rats and within this descending inhibitory pathway in both sexes is unknown. The present study used immunohistochemical techniques (1) to map the distribution of AR and ER $\alpha$  across the rostrocaudal axis of the PAG; and (2) to determine whether AR and/or ER $\alpha$  were colocalized on PAG neurons projecting to the RVM in male and female rats. AR and ER $\alpha$  immunoreactive neurons (AR-IR, ER $\alpha$ -IR) were densely distributed within the caudal PAG of male rats, with the majority localized in the lateral/ventrolateral PAG.

Females had significantly fewer AR-IR neurons, while the quantity of ER $\alpha$  was comparable between the sexes. In both sexes, approximately 25-50% of AR-IR neurons and 20-50% of ER $\alpha$ -IR neurons were retrogradely labeled. This study provides direct evidence of the expression of steroid receptors in the PAG and the descending pathway driving pain inhibition in both male and female rats and may provide a mechanism whereby gonadal steroids modulate pain and morphine potency.

April 24, 2008

To Whom It May Concern:

This manuscript complies with all contemporary standards of ethical practice and scientific publication regarding such matters study design and ethical approval, data provity and fabrication, authorship, declaration of conflict of interest, plaigerism and redundant publication.

Sincerely,  
Anne Z. Murphy  
Assoc. Professor



June 26, 2008

Dr. Harry W.M. Steinbusch, Editor-in-Chief  
*Journal of Chemical Neuroanatomy*  
European Graduate School of Neuroscience  
Department of Psychiatry and Neuropsychology  
Maastricht University  
Postbus 616, 6200 MD, Maastricht, The Netherlands

Dear Dr. Steinbusch:

We are resubmitting our manuscript [CHENEU-D-00030] entitled:

**" Androgen and Estrogen (a) Receptor Localization on Periaqueductal Gray – Rostral Ventromedial Medullary Neurons in the Male and Female Rat",**

for publication in *the Journal of Chemical Neuroanatomy*.

We were very pleased with the positive and thorough reviews of our manuscript, and are submitting a revised version including all major and minor changes as recommended by the three referees. The major changes are underlined in the text. A detailed list of all changes corresponding with each reviewers comments is given below.

Reviewer 1:

1. Sampling. For figure 2, on how many sections were cells counted from each rat for each level? One is not many. Was counting done blind to the treatment group? In cases where significant differences were found, can they be confirmed using repetitive sampling of number of cells per unit area (cell density), which may be more rigorous?

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percentile data to standard scores and conducted the ANOVA on these data. Fishers PLSD was used for post-hoc analysis.

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*Response:* The term "PAG-RVM" has been replaced throughout the manuscript with either "PAG neurons projecting to the RVM" or "PAG output neurons" where appropriate.

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*Response:* We now use 3 terms to more precisely describe the data: (1) -IR neurons that were retrogradely labeled; (2) the percentage of receptor that was localized in retrogradely labeled cells; and (3) the percentage of retrogradely labeled cells that expressed receptor.

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study (n was far too small/stage, and all females fell into only two stages). The inability to formally examine this variable given the present design should simply be acknowledged in the discussion.

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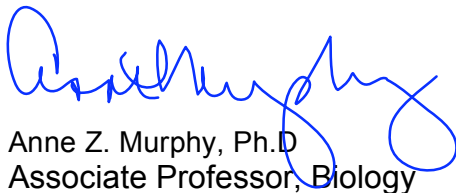
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*Response:* Figure 3 has been deleted as recommended.

In summary, we would like to thank the reviewers for taking the time and effort to review our manuscript. The reviews and comments were highly constructive, and we feel that the manuscript is much stronger with these revisions. We hope that you find our revised manuscript suitable for publication in the *Journal of Chemical Neuroanatomy*.

Sincerely,



Anne Z. Murphy, Ph.D.  
Associate Professor, Biology

## Detailed Response to Reviewers:

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Possible Reviewers

Rebecca M Craft, PhD  
Washington State Univ  
Dept Psychol  
PO BOX 644820  
Pullman WA 99164-4820  
Work Phone: 509-335-5040  
Fax: 509-335-5043  
E-mail Address: [craft@wsunix.wsu.edu](mailto:craft@wsunix.wsu.edu)

Richard J Bodnar, PhD  
Queens Col CUNY  
Dept Psychology  
65-30 Kissena Blvd  
Flushing NY 11367  
Work Phone: 718-997-3543  
Fax: 718-997-3257  
E-mail Address: [Richard\\_Bodnar@qc.edu](mailto:Richard_Bodnar@qc.edu)

Peggy Mason, PhD  
Univ Chicago  
Dept Neurobiol, Pharmacol & Physiol  
947 E 58th St, MC 0926  
Chicago IL 60637  
Work Phone: 773-702-3144  
Fax: 773-702-1216  
E-mail Address: [pmason@midway.uchicago.edu](mailto:pmason@midway.uchicago.edu)

Kathryn G Commons, PhD  
Children' Hosp, Harvard MS  
Anesthesiology  
300 Longwood Ave Enders 1070  
Boston MA 02115  
Work Phone: 617-919-2220  
Fax: 617-730-0199  
E-mail Address: [kathryn.common@childrens.harvard.edu](mailto:kathryn.common@childrens.harvard.edu)

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Dayna R. Loyd and Anne Z. Murphy

Department of Biology, Center for Behavioral Neuroscience  
Georgia State University, PO Box 4010, Atlanta, Georgia 30302-4010

Running Title: AR and ER $\alpha$  Localization on PAG-RVM Pathway

Correspondence to: Anne Z. Murphy, Ph.D.  
Dept. Biology,  
Center for Behavioral Neuroscience  
Georgia State University  
PO Box 4010  
Atlanta, GA 30303-4010  
amurphy@gsu.edu  
  
Phone: 404.413.5351  
Fax: 404.413.2509

Key Words: gonadal steroid receptor, hormone, immunohistochemistry, sex differences, descending modulatory pathway, pain, antinociception

Grant Support: NIH grants DA16272 and AR49555 to AZM

## ABSTRACT

The periaqueductal gray (PAG) is involved in many gonadal steroid-sensitive behaviors, including responsiveness to pain. The PAG projects to the rostral ventromedial medulla (RVM), comprising the primary circuit driving pain inhibition. Morphine administered systemically or directly into the PAG produces greater analgesia in male compared to female rats, while manipulation of gonadal hormones alters morphine potency in both sexes. It is unknown if these alterations are due to steroidal actions on PAG neurons projecting to the RVM. The expression of androgen (AR) and estrogen (ER $\alpha$ ) receptors in the PAG of female rats and within this descending inhibitory pathway in both sexes is unknown. The present study used immunohistochemical techniques (1) to map the distribution of AR and ER $\alpha$  across the rostrocaudal axis of the PAG; and (2) to determine whether AR and/or ER $\alpha$  were colocalized on PAG neurons projecting to the RVM in male and female rats. AR and ER $\alpha$  immunoreactive neurons (AR-IR, ER $\alpha$ -IR) were densely distributed within the caudal PAG of male rats, with the majority localized in the lateral/ventrolateral PAG. Females had significantly fewer AR-IR neurons, while the quantity of ER $\alpha$  was comparable between the sexes. In both sexes, approximately 25-50% of AR-IR neurons and 20-50% of ER $\alpha$ -IR neurons were retrogradely labeled. This study provides direct evidence of the expression of steroid receptors in the PAG and the descending pathway driving pain inhibition in both male and female rats and may provide a mechanism whereby gonadal steroids modulate pain and morphine potency.



## INTRODUCTION

Anatomical and physiological studies have shown that the midbrain periaqueductal gray (PAG) plays a modulatory role in a variety of behaviors including antinociception (Reynolds, 1969; Behbehani & Fields, 1979; Heinricher *et al.*, 1987; Behbehani, 1995; Budai *et al.*, 1998), reproduction (McCarthy *et al.*, 1991; Ogawa *et al.*, 1991; Murphy & Hoffman, 1998; Daniels *et al.*, 1999), fear and anxiety (Kim *et al.*, 1993), aggression (Bandler *et al.*, 1985; Bandler & Carrive, 1988; Depaulis *et al.*, 1992; Scordalakes & Rissman, 2004) and vocalization (Davis *et al.*, 1993; Zhang *et al.*, 1994). While these behaviors have been shown to be modulated by gonadal steroids, our knowledge of the qualitative and quantitative aspects of gonadal steroid receptors in the PAG is incomplete. The PAG has been shown to contain a large number of both androgen receptor (AR) and estrogen receptor (ER $\alpha$ ) immunoreactive neurons (Murphy & Hoffman, 1999; Murphy & Hoffman, 2001), however these studies were conducted exclusively in male rats. While the distribution of ER $\alpha$  in the female PAG has been reported in a few species, including the cat (VanderHorst *et al.*, 1998), the golden hamster (Boers *et al.*, 1999), the guinea pig (Turcotte & Blaustein, 1993) and the rhesus monkey (Vanderhorst *et al.*, 2002; VanderHorst *et al.*, 2004), the quantity and distribution of AR and ER $\alpha$  in the female rat is currently unknown.

The PAG projects heavily to the rostral ventromedial medulla (RVM), which in turn projects to the dorsal horn of the spinal cord. This PAG-RVM-spinal cord circuit is the primary neural pathway that elicits the antinociceptive effects of opiates. Previous studies have reported sex differences in the anatomical organization of the projections from the PAG to the RVM and activation of these neurons by inflammatory pain (Lloyd &

Murphy, 2006). In addition, there are significant sex differences in the activation of the this pathway by systemic morphine, both in the presence and absence of inflammatory pain (Loyd & Murphy, 2006; Loyd *et al.*, 2007; Loyd *et al.*, 2008). To date, it is not known whether ER $\alpha$  and AR are expressed on PAG neurons projecting to the RVM. Numerous behavioral studies have shown that sex differences in opioid analgesia are modulated by both the organizational and activational effects of gonadal steroids (Kepler *et al.*, 1989; Islam *et al.*, 1993; Krzanowska & Bodnar, 1999; 2000; Stoffel *et al.*, 2003; Cataldo *et al.*, 2005; Stoffel *et al.*, 2005). Male rats castrated at birth experience decreased morphine potency in adulthood, while female rats masculinized at birth experience greater morphine potency in adulthood whether morphine is administered systemically (Cicero *et al.*, 2002) or directly into the PAG (Krzanowska *et al.*, 2002). Similarly, both systemic and central administration of morphine is less effective in gonadectomized adult males and more effective in ovariectomized adult females (Kepler *et al.*, 1989; Ratka & Simpkins, 1991; Krzanowska & Bodnar, 1999; Turner *et al.*, 2002; Stoffel *et al.*, 2003; Stoffel *et al.*, 2005; Turner *et al.*, 2005); effects are reversed with hormone replacement (Ratka & Simpkins, 1991; Kiefel & Bodnar, 1992); Stoffel *et al.*, 2003; (Ji *et al.*, 2007).

While the organizational and activational effects of gonadal steroids are likely to contribute to the sexually dimorphic actions of morphine, it is currently unknown whether gonadal steroid receptors are expressed on PAG neurons projecting to the RVM. In addition, the qualitative and quantitative aspects of AR and ER $\alpha$  expression in the PAG of the female rat are not known. The present studies utilized immunohistochemistry to map (1) the quantity and distribution of AR and ER $\alpha$  immunoreactive neurons across

the rostrocaudal axis of the PAG; and (2) to determine if the PAG neurons projecting to the RVM express AR and ER $\alpha$  immunoreactivity. Due to a lack of commercially available antibodies at the time these studies were conducted, ER $\beta$  was not analyzed in this study. This study is the first to report AR and ER $\alpha$  immunoreactivity in the PAG and its descending projections to the RVM in both male and female rats.

## **MATERIALS AND METHODS**

### *Subjects*

Six adult male and six weight-matched (250-350g; approximately 70-100 days of age) cycling female Sprague-Dawley rats were used in these experiments (Zivic-Miller; Pittsburgh, PA). Rats were housed in same-sex pairs on a 12:12 hour light:dark cycle. Access to food and water was ad libitum throughout the experiment except during surgery. These studies were performed in compliance with the Institutional Animal Care and Use Committee at Georgia State University. All efforts were made to reduce the number of animals used in these experiments and to minimize any possible suffering by the animal.

### *Vaginal Cytology*

Vaginal lavages were performed daily beginning two weeks prior to experimental manipulations to confirm that the female rats were cycling normally and to keep daily records on the stages of their cycle up to the day of sacrifice. Proestrus was identified as a predominance of nucleated epithelial cells and estrus was identified as a predominance of cornified epithelial cells. Diestrus 1 was differentiated from Diestrus 2

by the presence of leukocytes. Rats that appeared between phases were noted as being in the more advanced stage.

### *Retrograde Tracer Injections*

Animals were deeply anesthetized with a cocktail of ketamine/xylazine/acepromazine (50 mg/kg / 3.3 mg/kg / 3.3 mg/kg; i.p.; Henry Schein, Melville, NY). When a surgical plane of anesthesia was reached each animal was placed in a stereotaxic frame and the skull was adjusted so bregma and lambda were at the same dorsal-ventral plane. Glass micropipettes (10-20  $\mu$ M) filled with the retrograde tracer Fluorogold (FG; 2% soln. w/v in saline; Fluorochrome LLC; Denver, CO) were lowered into the RVM using the following coordinates (in mm): AP: -2.0 Lambda; ML: 0.0; DV: -8.5). FG was iontophoresed (50/50 duty cycle, 7.5  $\mu$ A current) into the RVM for 25 minutes to facilitate neuronal uptake. The current was then turned off and the pipettes remained in place for an additional 5 minutes prior to removal to minimize backflow of the tracer along the pipette track. Following tracer injections, wounds were sutured closed, the antibiotic Neosporin was applied to the wound, and the animals were placed in clean cages to recover under a heat lamp. Upon complete recovery from the anesthetic, animals were returned to their original housing facilities.

### *Perfusion fixation*

Ten days following surgery, animals were given a lethal dose of Nembutal (160 mg/kg; i.p.) and transcardially perfused with 200-250 ml of 0.9% sodium chloride containing 2% sodium nitrite as a vasodilator to remove blood from the brain. Immediately following removal of blood, 300 ml of 4% paraformaldehyde in 0.1M phosphate buffer containing 2.5% acrolein (Polyscience; Niles, IL) was perfused

through the brain as a fixative. A final rinse with 200-250 ml of the sodium chloride/sodium nitrate solution was perfused through the brain to remove any residual acrolein. Immediately following perfusion, the brains were carefully removed, placed in a 30% sucrose solution and stored at 4°C for at least one week prior to sectioning. Sucrose solutions were changed daily to optimize saturation of sucrose into the tissue. To section the brain, the dura and pia mater were carefully removed and the brains were cut into six series of 25 µm coronal sections with a Leica 2000R freezing microtome and stored free-floating in cryoprotectant-antifreeze solution (Watson et al., 1986) at -20°C until immunocytochemical processing. The tissue was sectioned at 25 µm so that 125 µm separates each analyzed level of the PAG thus eliminating any possible bias from counting the same cell twice during data collection.

### *Immunocytochemistry*

A 1:6 series through the rostrocaudal axis of each brain was processed for FG immunoreactivity and AR (n=5 males; n=6 females) or ERα (n=6 males; n=5 females) immunoreactivity as previously described (Murphy & Hoffman, 2001). Briefly, sections were rinsed extensively in potassium phosphate-buffered saline (KPBS) to remove cryoprotectant solution, immediately followed by a 20-minute incubation in 1% sodium borohydride to remove excess aldehydes. The tissue was then incubated in either primary antibody solution rabbit anti-AR (Santa Cruz Biotechnology; Santa Cruz, CA, lot no. L0407; 1:10,000) or rabbit anti-ERα (Santa Cruz Biotechnology; Santa Cruz, CA, lot no. I2607; 1:20,000) in KPBS containing 1.0% Triton-X for one hour at room temperature followed by 48 hours at 4°C. The rabbit anti-AR antiserum was prepared against a peptide mapping at the N-terminus of AR of human origin

(MEVQLGLGRVYPRPPSKTYRG) corresponding to amino acids 2-21 (manufacturer's technical information) and specificity has been confirmed (Creutz & Kritzer, 2004). The rabbit anti-ER $\alpha$  antiserum was prepared against a peptide mapping at the C-terminus of ER $\alpha$  of mouse origin (HSLQTYIIPPEAEGFPNTI) corresponding to amino acids 580-559 (manufacturer's technical information) and specificity has been confirmed (Quesada *et al.*, 2007).

After rinsing out the primary antibody with KPBS, the tissue was incubated for one hour in biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch; West Grove, PA, 1:600), rinsed with KPBS, followed by a one hour incubation in an avidin-biotin peroxidase complex (1:10; ABC Elite Kit, Vector Labs). After rinsing in KPBS and sodium acetate (0.175 M; pH 6.5), AR or ER $\alpha$  immunoreactivity was visualized as a black reaction product using nickel sulfate intensified 3,3'-diaminobenzidine solution containing 0.08% hydrogen peroxide in sodium acetate buffer. After rinsing, AR or ER $\alpha$  labeled sections were then placed in primary antibody solution rabbit anti-FG (Chemicon; Billerica, MA, lot no. 25060005; 1:10,000) in KPBS containing 1.0% Triton-X for one hour at room temperature followed by 48 hours at 4°C. FG was visualized as a brown reaction product using 3,3'-diaminobenzidine containing 0.08% hydrogen peroxide in Trizma buffer (pH 7.2). After 15-30 minutes, three rinses in sodium acetate buffer terminated the reaction and tissue was given a final rinse in KPBS. Sections were then mounted out of saline onto gelatin-subbed slides, air-dried and dehydrated in a series of graded alcohols. Tissue-mounted slides were then cleared in xylene and glass cover-slipped using Permount.

*Data Analysis and Presentation*

Data were analyzed across six representative levels through the rostrocaudal axis of the PAG (Bregma -6.72, -7.04, -7.74, -8.00, -8.30, -8.80). The number of AR immunoreactive neurons (AR-IR), ER $\alpha$  immunoreactive neurons (ER $\alpha$ -IR), and the number of AR-IR and ER $\alpha$ -IR neurons that were retrogradely labeled (AR/FG+, ER $\alpha$ /FG+) were quantified. The experimenter was blind to the experimental condition. In levels where significant differences were found, a second blinded observer confirmed results. Cell counts were conducted unilaterally as there are no differences in the number of FG+ cells (Loyd & Murphy, 2006) or the number of AR-IR and ER $\alpha$ -IR neurons (Murphy & Hoffman, 2001) for the left versus right side of PAG. Additionally, previous data have shown that there are no sex differences in total area (mm<sup>2</sup>) of the PAG between weight-matched male and female Sprague-Dawley rats (Loyd & Murphy, 2006).

Data are reported as the mean  $\pm$  standard error of the mean (SEM) from which percentages were calculated and reported as the percentage of receptor that was localized in retrogradely labeled cells (%AR/FG+; %ER $\alpha$ /FG+) or as the percentage of retrogradely labeled cells that were colocalized with receptor (%FG/AR; %FG/ER $\alpha$ ). A three-way analysis of variance (ANOVA) was used to test for significant main effects of sex (male, female), PAG level (Bregma -6.72 through -8.80), and PAG subdivision (dorsomedial, lateral/ventrolateral). For percentile data, percentages were transformed to standard scores. Fishers's post hoc tests were used to determine specific group differences when a main effect or interaction was observed.  $P \leq .05$  was considered significant for all analyses. For data presentation, a representative animal from each

experimental group was selected and the distribution of (1) AR-IR neurons, (2) ER $\alpha$ -IR neurons, (3) FG+ neurons, (4) AR/FG+ neurons and (5) ER $\alpha$ /FG+ neurons within the PAG were plotted using a Nikon Drawing Tube attached to a Nikon Optiphot microscope. Plots were then scanned onto the computer and adjusted to figure format using Adobe Illustrator 10. Photomicrographs were generated using a Synsys digital camera attached to a Nikon Eclipse E800 microscope. Images were captured with IP Spectrum software and adjusted to figure format by alterations in brightness and contrast levels using Adobe Photoshop 7.0.

## RESULTS

### *Androgen Receptor Distribution in the PAG*

AR-IR neurons were distributed across the rostrocaudal axis of the PAG in both male and female rats (Figure 1; red circles). AR-IR neurons were confined to the dorsomedial and lateral/ventrolateral subdivisions of the PAG, with the dorsolateral subdivision of the PAG lacking AR-IR neurons. These results are consistent with previous studies showing AR localization in the PAG of male rats (Murphy & Hoffman, 1999; Murphy & Hoffman, 2001). While the qualitative distribution of AR-IR neurons was similar in both males and females, quantitatively males had a significantly greater number of AR-IR neurons compared to females [ $F(1, 54)=22.7$ ,  $p<.00001$ ] (Figure 2; red bars) with a significantly greater number of AR-IR neurons localized in the lateral/ventrolateral PAG compared to the dorsomedial subdivision [ $F(1, 108)=22.1$ ,  $p<.0001$ ]. This sex difference was evident across the rostrocaudal axis of the PAG. There was no main effect of level of PAG [ $F(5,54)=1.2$ ; n.s.] and no significant sex by



level interaction [ $F(5,54)=0.3$ ; n.s.], indicating that the number of AR-IR neurons remained consistent across the rostrocaudal axis of the PAG of both male and female rats.

#### *Estrogen ( $\alpha$ ) Receptor Distribution in the PAG*

ER $\alpha$ -IR neurons were densely distributed throughout the rostrocaudal axis of the PAG in both male and female rats (Figure 1; black circles). Similar to the distribution of AR-IR neurons, ER $\alpha$ -IR neurons were confined to the dorsomedial and lateral/ventrolateral subdivisions of the PAG, with the majority of ER $\alpha$ -IR neurons localized in the lateral/ventrolateral PAG [ $F(1, 108)=105.8$ ,  $p<.0001$ ]. Overall, there was no sex difference in the number of ER $\alpha$ -IR neurons in the PAG [ $F(1, 54)=1.2$ , n.s.] (Figure 2; black bars). A significant increase in the number of ER $\alpha$ -IR neurons [ $F(5, 54)=9.02$ ,  $p<.0001$ ] was noted along the rostrocaudal axis of the PAG.

#### *Androgen Receptor Distribution in PAG Neurons Projecting to the RVM*

All iontophoretic injections of the retrograde tracer Fluorogold (FG) into the RVM were located on the midline and dorsal to the pyramidal tract, at the level of the caudal pole of the facial nucleus (lambda  $-2.0$ mm). Analysis was limited to injection sites that occurred between the facial nucleus and the olivary complex across approximately 2mm rostrocaudally (Bregma  $-9.30$  to  $-11.60$ ). In our studies using anterograde tracing from the PAG to the RVM, we have noted that this region of RVM contains the highest density of anterogradely labeled fibers and is remarkably consistent throughout this 2mm window of RVM (unpublished observations). Injections outside of the RVM were not included for analysis. Only male and female rats with comparable injection sites were used for analysis. Injection of FG into the RVM produced dense retrograde

labeling throughout the rostrocaudal axis of the PAG consistent with our previous studies (Loyd et al, 2006, Loyd et al., 2007; Loyd et al., 2008). Females had a significantly greater number of PAG cells retrogradely labeled from the RVM compared to males [ $F(1,70)=14.4$ ,  $p<.0003$ ].

Figure 3 shows an example of AR and FG immunoreactivity within the lateral PAG of a representative male (A-B) and female (C-D) rat. AR-IR neurons there were retrogradely labeled were densely localized throughout the rostrocaudal axis of the PAG in both male and female rats (Figure 4; red stars), with males expressing more dual labeled cells [ $F(1,54)=19.5$ ;  $p<.0001$ ]. The percentage of retrogradely labeled cells that expressed AR was comparable between the sexes [ $F(1,54)=13.51$ ; n.s.] (Figure 5, %FG/AR), and significantly increased moving caudally through the PAG [ $F(5,54)=7.29$ ;  $p<.0001$ ]. Since female rats had a greater number of PAG neurons projecting to the RVM compared to males, the percentage of AR that was localized in retrogradely labeled cells was also determined (Figure 5; %AR/FG+) and was found to be comparable between the sexes [ $F(1, 54)=1.4$ ; n.s.].

#### *Estrogen ( $\alpha$ ) Receptor Distribution in PAG Neurons Projecting to the RVM*

An example of ER $\alpha$  and FG immunoreactivity within the lateral PAG of a representative male (A-B) and female (C-D) rat is shown in Figure 6. ER $\alpha$ -IR neurons that were retrogradely labeled were densely localized throughout the rostrocaudal axis of the PAG in both male and female rats (Figure 4; open stars) [ $F(1,54)=1.1$ ; n.s.], with the majority of localized in the lateral/ventrolateral subdivision [ $F(1, 54)=7.5$ ;  $p<.0001$ ]. Across all levels and regions of the PAG, the percentage of retrogradely labeled cells that expressed ER $\alpha$  was comparable between the sexes [ $F(1,54)=0.176$ ; n.s.] (Figure 5,

%FG/ER $\alpha$ ). Similarly, the percentage of ER $\alpha$  that was localized in retrogradely labeled cells was comparable between the sexes [ $F(1, 54)=0.292$ ; n.s.] (Figure 5; %ER $\alpha$ /FG+). Additionally, a significantly greater percentage of retrogradely labeled cells that expressed ER $\alpha$  were observed in the caudal PAG [ $F(5,54)=15.1$ ;  $p<.0001$ ].

## DISCUSSION

The PAG has been implicated in a variety of hormone-sensitive behaviors (Bandler & Shipley, 1994; Keay & Bandler, 2001; 2002); however, gonadal steroid receptor expression in the PAG had not been reported in both male and female rats. Here we report that the expression of AR is sexually dimorphic along the entire rostrocaudal axis of the PAG, with males having a significantly greater number of immunoreactive neurons. No sex differences were noted in the qualitative or quantitative aspects of ER $\alpha$  expression in the PAG. In the present study, ER $\beta$  was not examined due to a lack of a reliable antibody at the time these studies were conducted; therefore, the possibility remains that the expression of ER $\beta$  in the PAG is sexually dimorphic. Similarly, in this study we were unable to determine the effects of estrous on ER $\alpha$  expression in the PAG due to low number of animals. On the day of sacrifice, ten days following tracer injections, all female rats were in either the estrus ( $n=4$ ) or proestrus ( $n=2$ ) phase of their cycle; no animals were in the diestrus phase.

The sex difference in the expression of AR in the PAG may play a role in sex differences in pain and analgesia. There are numerous behavioral studies indicating a role of gonadal steroids in modulating morphine potency. Gonadectomy reduces morphine potency in male rats (Kepler et al., 1989) and increases morphine potency in

females (Turner *et al.*, 2002; Turner *et al.*, 2005), while hormone replacement reverses these effects (Stoffel *et al.*, 2003; Stoffel *et al.*, 2005). Masculinizing female rat pups with testosterone increases morphine potency to male-like levels (Cicero *et al.*, 2002). In addition, testosterone has been shown to oppose the effects of estradiol on neuronal excitability (Edwards *et al.*, 1999) and decrease pain sensitivity in both male and female rats (Aloisi *et al.*, 2004). A greater expression of AR in the PAG of males may provide an anatomical substrate for the sexually dimorphic modulation of pain by gonadal steroids.

The distribution of AR-IR and ER $\alpha$ -IR neurons was remarkably similar; both receptor types were preferentially localized within the dorsomedial and lateral/ventrolateral subdivisions of the PAG and both increased in density along the rostrocaudal axis of the PAG. These results are similar to the distribution of AR-IR and ER $\alpha$ -IR neurons previously reported in the PAG of the male rat (Murphy & Hoffman, 1999; Murphy & Hoffman, 2001). In addition, the distribution of ER $\alpha$ -IR neurons in the female rat PAG is similar to that previously reported in the cat (VanderHorst *et al.*, 1998), the golden hamster (Boers *et al.*, 1999), the guinea pig (Turcotte & Blaustein, 1993) and the rhesus monkey (Vanderhorst *et al.*, 2002; VanderHorst *et al.*, 2004).

#### *Steroid Receptor Colocalization within the Endogenous Descending Pathway Driving Pain Inhibition*

The dense projections from the PAG to the RVM provide an essential neural circuit for the antinociceptive effects of opiates. Many behavioral studies have reported an effect of steroid hormones on morphine potency; however, this study is the first to report the expression of AR and ER $\alpha$  within the endogenous descending pathway

driving pain inhibition. Using immunohistochemical analysis, we report that AR and ER $\alpha$  were expressed on PAG neurons projecting to the RVM in both the dorsomedial and lateral/ventrolateral subdivisions of the PAG. Male rats had a greater number AR-IR neurons that were retrogradely labeled, however, there was no sex difference in either the percentage of retrogradely labeled cells that expressed AR or the percentage of AR that was located within retrogradely labeled cells. Similarly, the percentage of ER $\alpha$  that was localized in retrogradely labeled cells was comparable between the sexes and was significantly greater in the caudal PAG with the majority localized in the lateral/ventrolateral subdivision.

#### *Role in Pain and Analgesia*

The present results report a dense colocalization of gonadal steroid receptors on PAG neurons projecting to the RVM, which may provide the anatomical substrate for the reported sex differences in morphine potency. Between 27-50% of PAG neurons projecting to the RVM contain mu opioid receptor (MOR); these MOR+ cells are localized primarily within the caudal lateral/ventrolateral PAG (Commons *et al.*, 2000; Wang & Wessendorf, 2002), in the same subdivision of the PAG that we report a dense distribution of both steroid hormone receptors. Estradiol has been shown to both uncouple MORs from G protein-gated inwardly rectifying potassium channels causing a reduction in hyperpolarization by MOR agonists (Kelly *et al.*, 2003) and induce mu opioid receptor (MOR) internalization (Eckersell *et al.*, 1998). Furthermore, MOR internalization requires the presence of ER $\alpha$  (Micevych *et al.*, 2003) suggesting that colocalization of MOR and ER $\alpha$  in the descending inhibitory circuit may provide a

mechanism through which gonadal hormones differentially affect morphine potency in male and female rats.

Although not determined in the present study, it is possible that both AR and ER $\alpha$  are colocalized within the same PAG cells, as is the case in other brain areas (Wood & Newman, 1995); (Greco *et al.*, 1998). A population of neurons expressing both AR and ER $\alpha$  in the PAG may provide a potential mechanism for the diverse effects of gonadal steroid hormones. For example, there are numerous reports of sex differences in pain sensitivity; however, there is no clear consensus on the direction of the sex difference (Mogil *et al.*, 2000; Gaumond *et al.*, 2002; Aloisi *et al.*, 2004; LaCroix-Fralish *et al.*, 2005). In addition, pain sensitivity varies across the rat estrous cycle (Gintzler, 1980) and the human menstrual cycle (Cogan & Spinnato, 1986; Hellstrom & Anderberg, 2003). Sex differences in circulating gonadal steroids acting via a differential expression of AR and ER $\alpha$  within the same PAG neuron may provide a mechanism for the diverse effects of gonadal steroids on pain sensitivity.

#### *Other Functional Considerations*

The PAG has also been implicated in the regulation of the autonomic system controlling blood pressure, heart rate, and regional blood flow, all of which have been shown have shown to be modulated by gonadal hormones (Alper & Schmitz, 1996); (Morgan & Pfaff, 2001). In parallel, the PAG initiates defensive and aggressive behaviors, such as the 'fight or flight' response (Bandler *et al.*, 1985; Bandler & Carrive, 1988; Depaulis *et al.*, 1992; Scordalakes & Rissman, 2004), and evidence suggests that gonadal hormones increase these behaviors in both male and female rats (Albert *et al.*, 1990; Albert *et al.*, 1991; Johansson *et al.*, 2000). Additionally, the PAG has also been

implicated in initiating sex behavior, in that stimulation of the PAG facilitates lordosis in female rats (Sakuma & Pfaff, 1979a; 1979b; McCarthy *et al.*, 1991), while lesions of the PAG suppress this behavior (Sakuma & Pfaff, 1979b; Lonstein & Stern, 1998). Here we report that the PAG, an anatomical substrate essential for the integration of sensory input and autonomic output, contains a large population of gonadal steroid receptor-expressing neurons, which appear to be involved in modulating both autonomic and sensory responses involved in producing steroid-sensitive behaviors.

### *Summary*

The present study demonstrates that there are sex differences in the qualitative and quantitative aspects of the gonadal steroid receptors in the PAG. These reported differences in AR and ER $\alpha$  immunoreactivity in the PAG have an important impact on steroid-sensitive behaviors modulated by the PAG, such as reproduction, aggression, and autonomic regulation. We additionally report that the primary neural circuit for the antinociceptive effects of opioids expresses steroid receptors and may provide a direct mechanism for sex differences in morphine analgesia.

## ACKNOWLEDGMENTS

The authors would like to acknowledge the technical assistance of Leslie Bush on data collection. This work was supported by NIH grants DA16272 and P50 AR49555 awarded to Anne Z. Murphy, Ph.D.



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## TITLES AND LEGENDS TO FIGURES

**Figure 1.** Distribution of cells in the PAG immunoreactive for AR (red circles) and ER $\alpha$  (black circles) in male (left side of plots) and female rats (right side of plots) at six rostrocaudal levels (A-F) of the PAG.

**Figure 2.** Bar graphs display the mean number ( $\pm$  S.E.M.) of AR immunoreactive cells (green bars) and ER $\alpha$  immunoreactive cells (purple bars) across six rostrocaudal levels of the PAG. Cell counts were combined for the dorsomedial and lateral/ventrolateral subdivisions of PAG. # denotes a significant sex difference in mean # of steroid receptors.

**Figure 3.** Color photomicrograph showing a low (A,C) and high (B,D) power example of single- and double-labeled AR and FG immunoreactive cells in the lateral PAG (bregma -8.00) of a male (A-B) and female rat (C-D). Scale bar = 100  $\mu$ m for low power images; scale bar = 50  $\mu$ m for high power images.

**Figure 4.** Distribution of cells in the PAG retrogradely labeled from the rostral ventromedial medulla (black circles) and immunoreactive for AR (red stars) and ER $\alpha$  (open stars) in male (left side of plots) and female rats (right side of plots) at six rostrocaudal levels of the PAG.

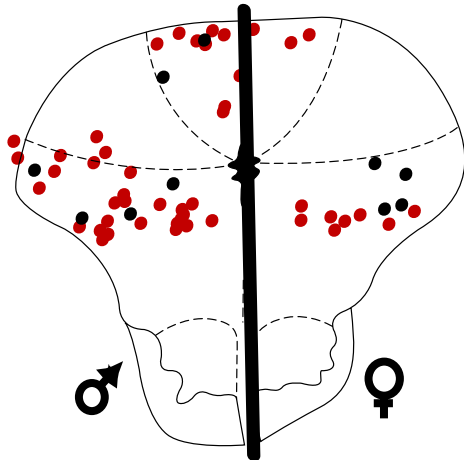
**Figure 5.** Bar graphs display the mean ( $\pm$  S.E.M.) %AR/FG+, %FG/AR+, %ER $\alpha$ /FG+, and %FG/ER $\alpha$ + immunoreactive neurons for the dorsomedial combined with lateral/ventrolateral regions of PAG across six rostrocaudal levels of the PAG.

**Figure 6.** Color photomicrograph showing a low (A,C) and high (B,D) power example of single- and double-labeled ER $\alpha$  and FG immunoreactive cells in the lateral PAG

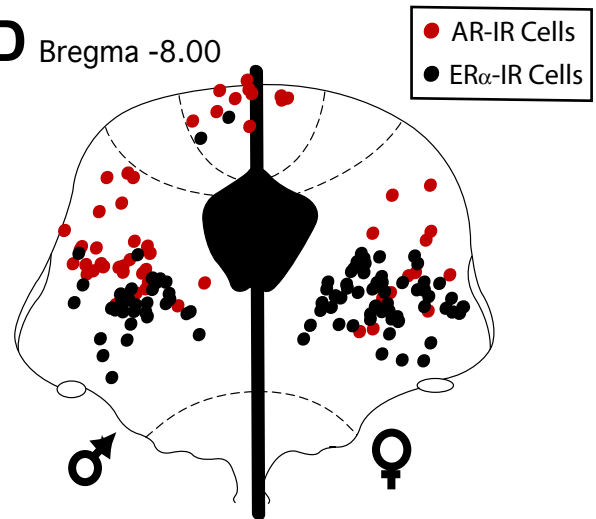
(bregma -8.00) of a male (A-B) and female rat (C-D). Scale bar = 100  $\mu\text{m}$  for low power images; scale bar = 50  $\mu\text{m}$  for high power images.



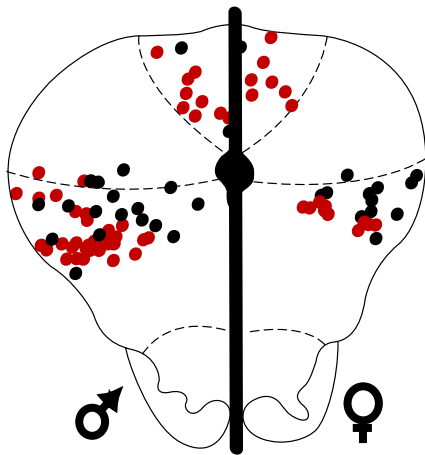
**A** Bregma -6.72



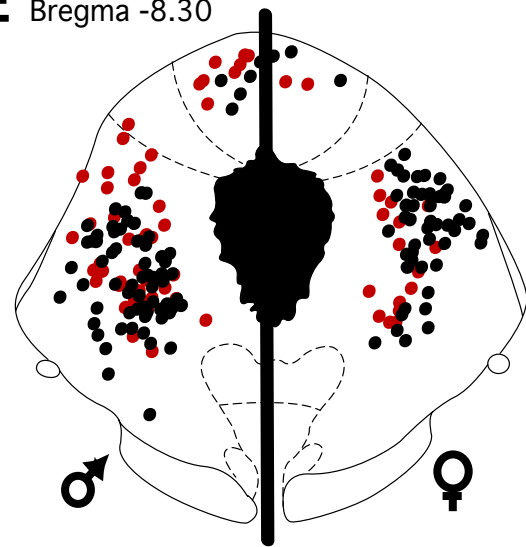
**D** Bregma -8.00



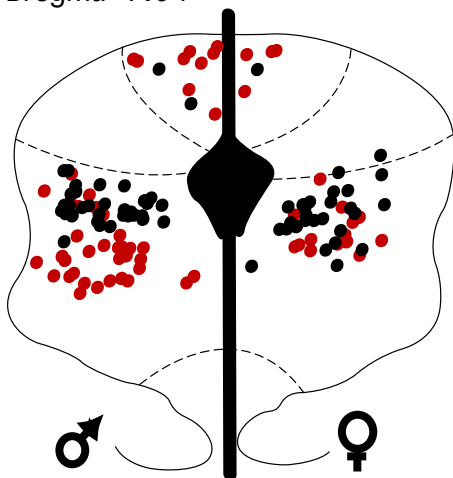
**B** Bregma -7.04



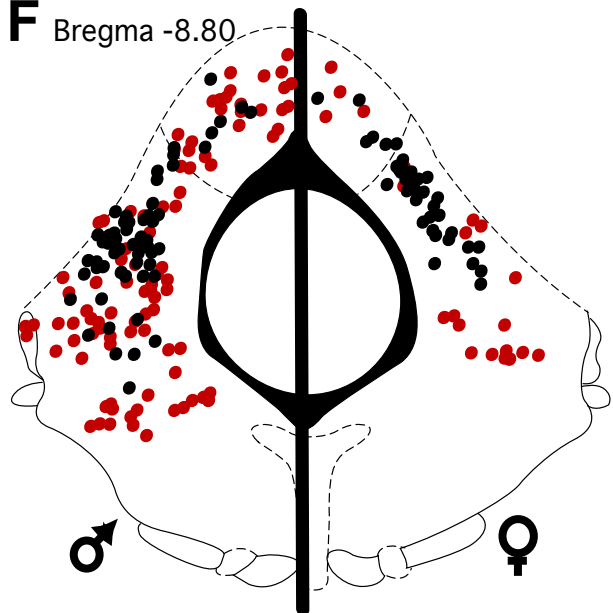
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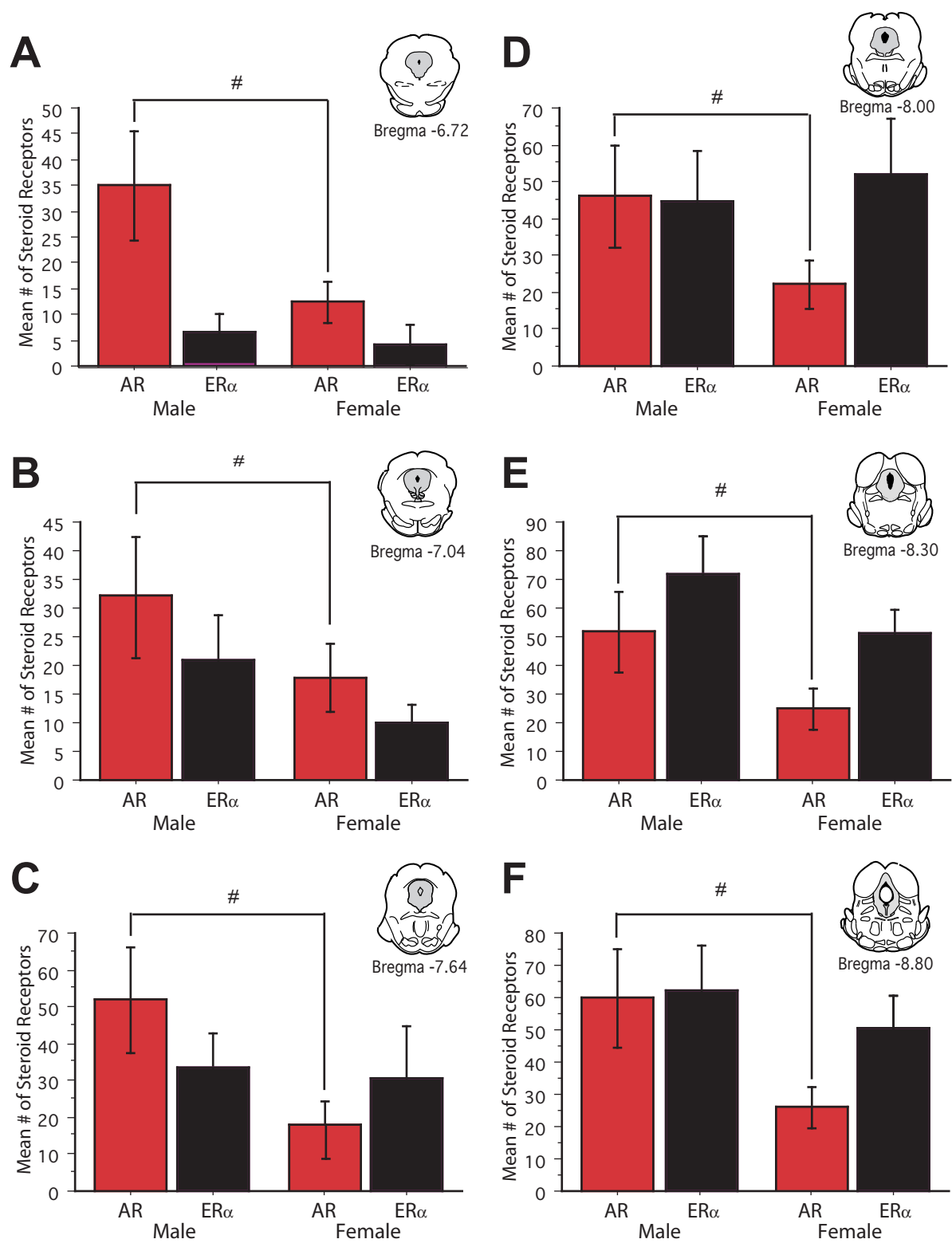
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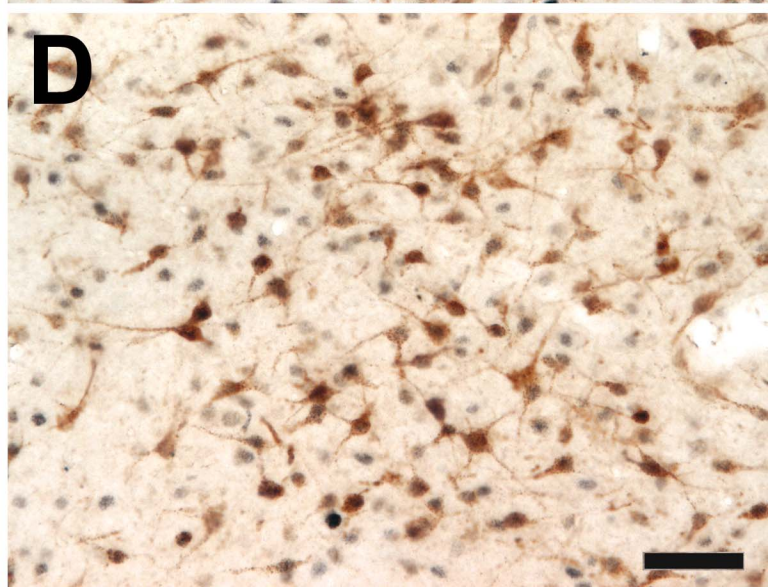
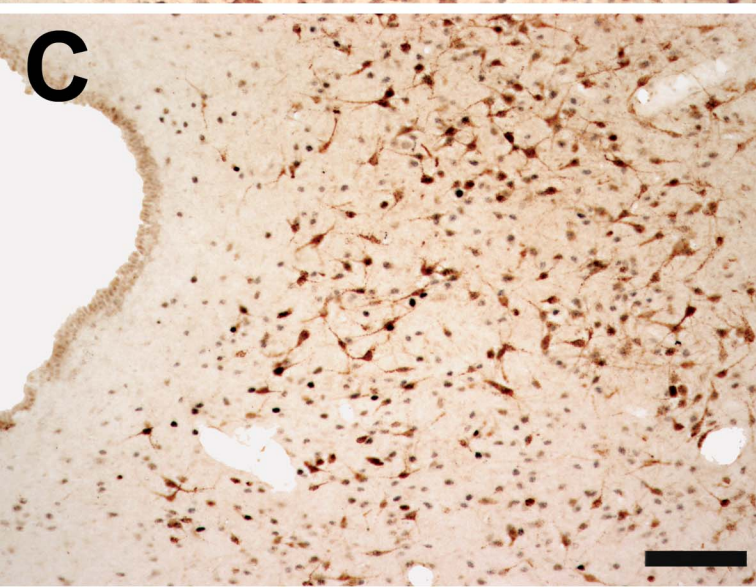
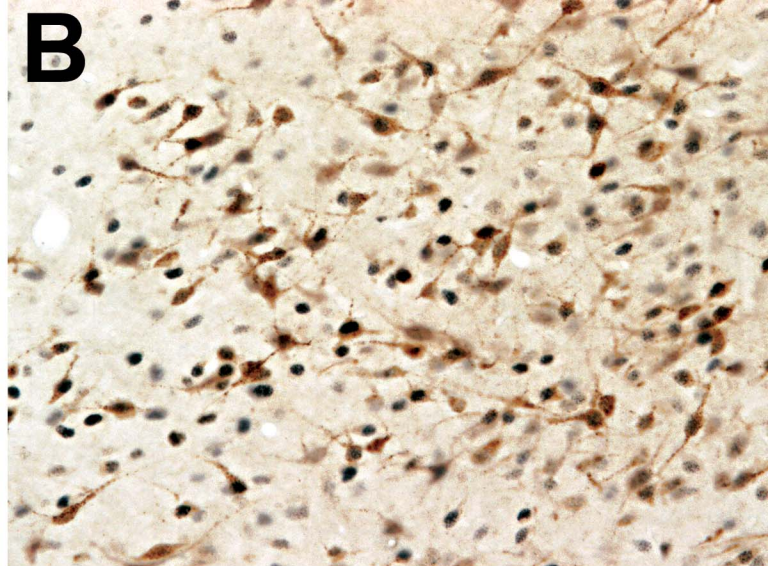
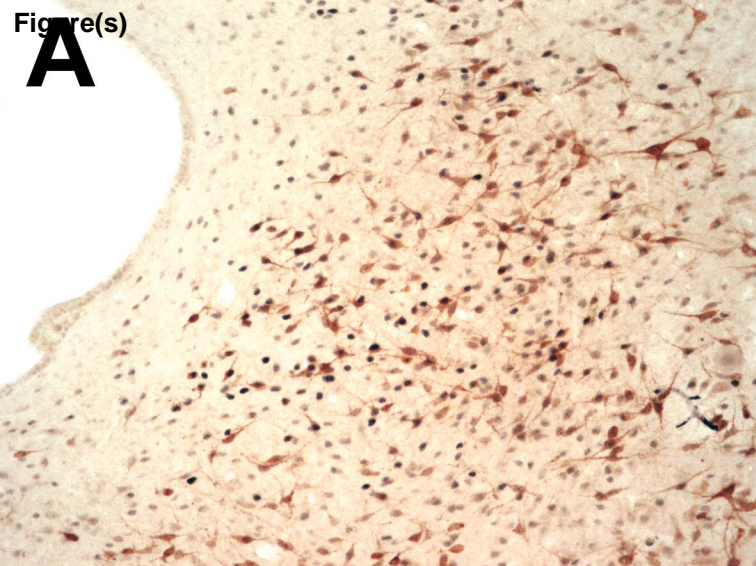
**F** Bregma -8.80



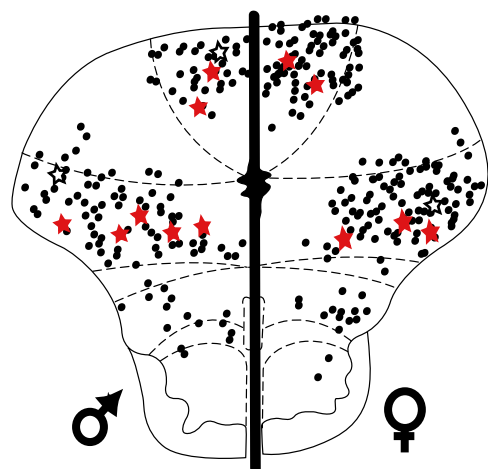
Figure(s)



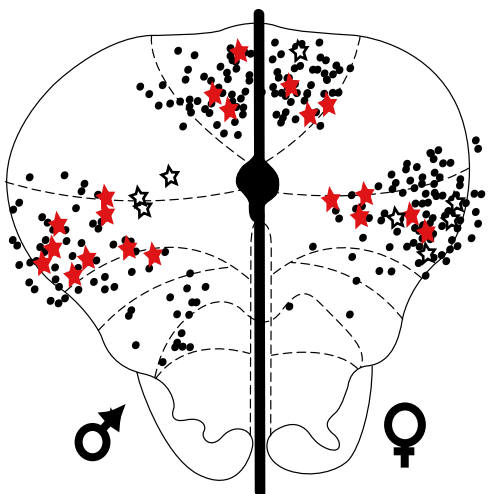
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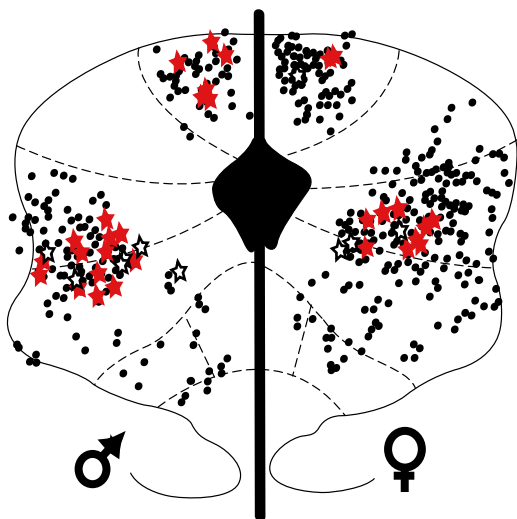
**A** Bregma -6.72



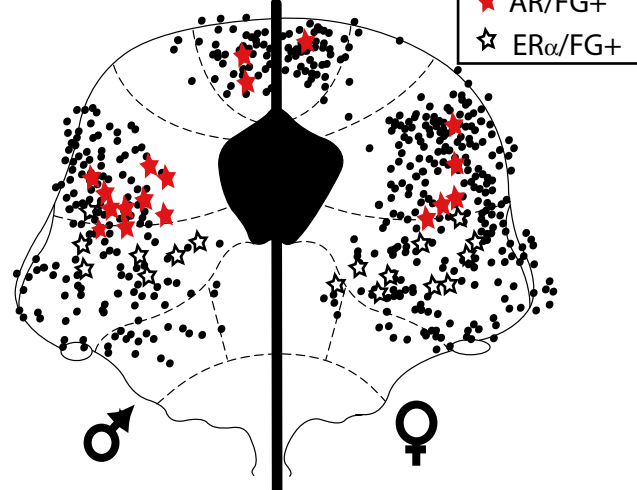
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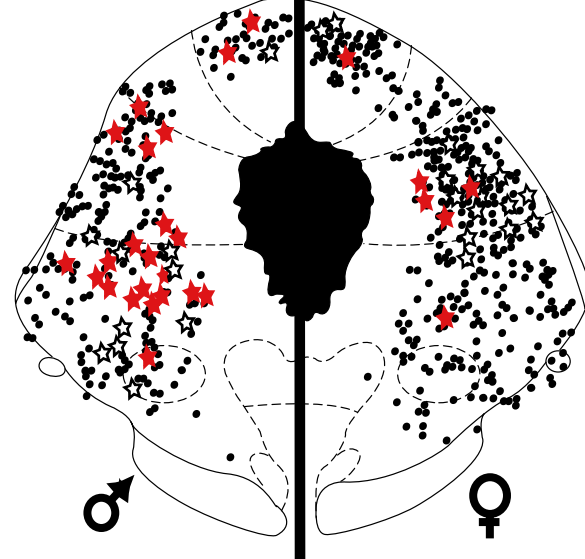
**C** Bregma -7.64



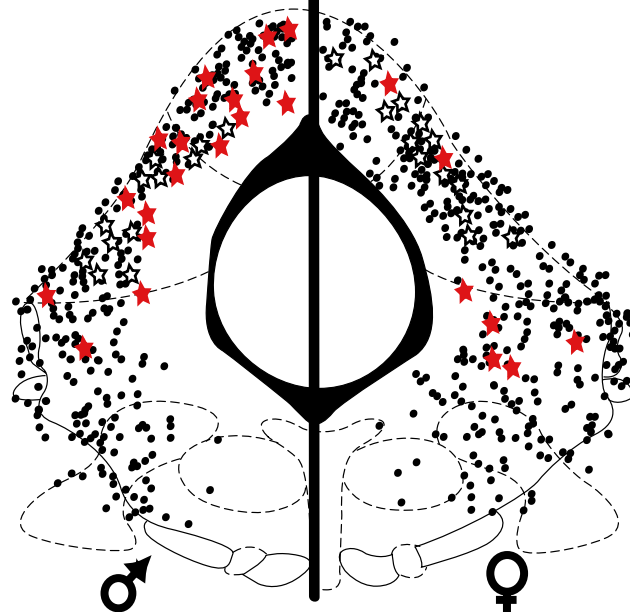
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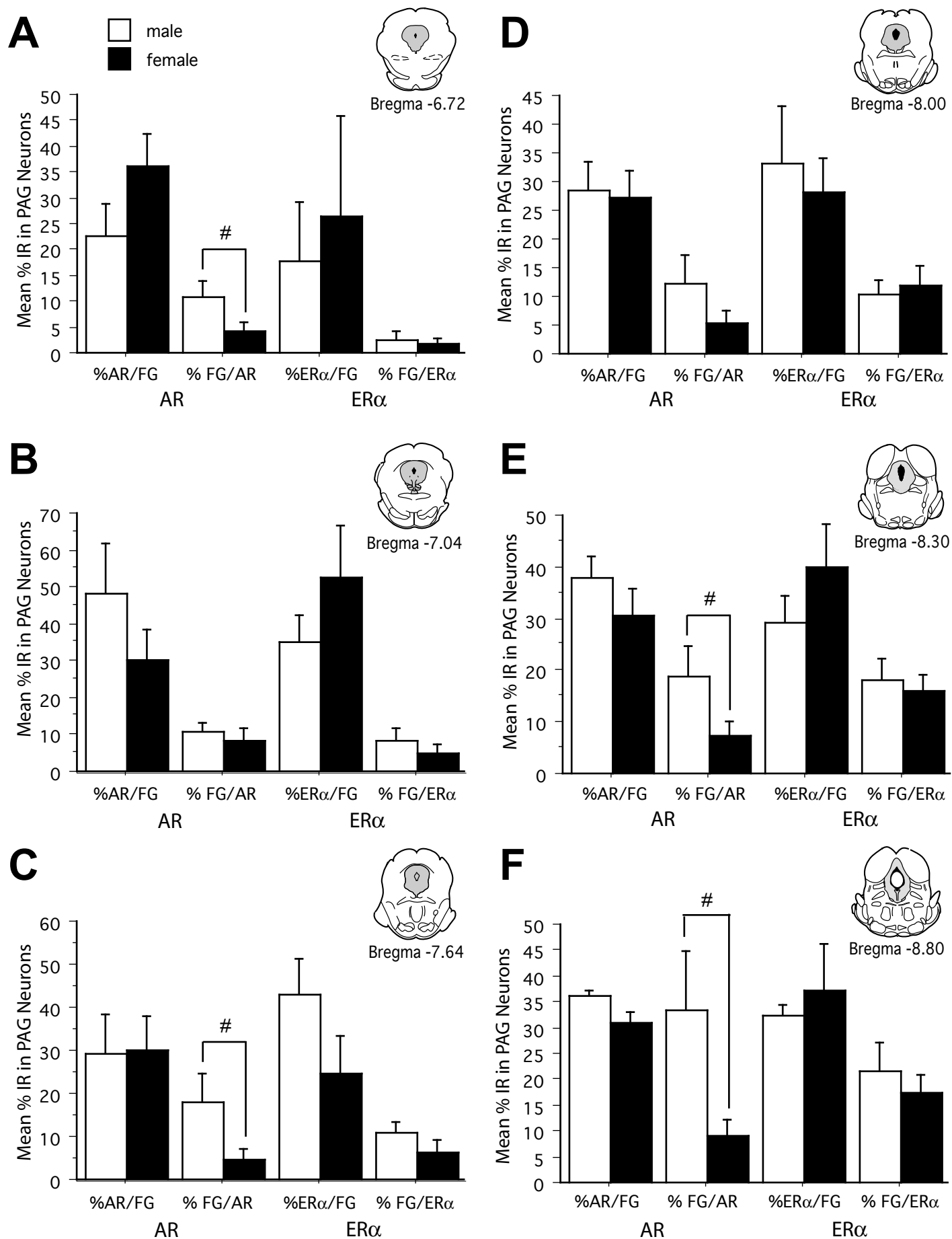
**E** Bregma -8.30



**F** Bregma -8.80



Figure(s)





Figure(s)

